

Counterion collapse and the effect of diamines on bacteriorhodopsin

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A recent report of electrical measurements on oriented bacteriorhodopsin in gels [(1986) FEBS Lett. 195, 164-168] concluded that low concentrations of diamines reversed the direction of the proton pump. Calculations are presented which show that in low diamine concentrations, charge displacements of the counterion atmosphere in the direction opposite to proton pumping are expected following H^+ ejection. It is also shown that the effect will be sharply reduced by raising the diamine concentration or by adding excess salt, as was observed. Hence it is not necessary to conclude that diamines reverse the direction of the proton pump itself.

Bacteriorhodopsin; Purple membrane; Proton pump; Counterion; Surface charge; Diamine

1. INTRODUCTION

Bacteriorhodopsin (bR) is the retinal-protein located in the purple membrane (PM) of *Halobacterium halobium* which functions as a light-driven proton pump [1,2]. Time-resolved electrical measurements of PM fragments adsorbed onto planar lipid bilayers [3], thin teflon [4] or collodion films [5] indicate rapid charge movements within the bR after a light flash. Similar results have been obtained using a novel method in which the PM fragments were oriented in a weak d.c. electric field [6,7] or in gels in which the oriented PM was immobilized [8]. Recently, it was reported [9] that the direction of the proton pump was reversed in the presence of low concentrations of diamines such as *N,N,N',N'*-tetramethylethylenediamine (TEMED). This was inferred from the apparent change in direction of displacement currents in oriented PM gels. Simple monoamines had no such effect.

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I wish to suggest another interpretation for the data in [9]. Specifically, on the time scale relevant to proton release into the external aqueous phase, the change in the displacement currents reflects a shift in the counterion atmosphere near the highly negatively charged PM surface. Quantitative analysis using numerical integration of the Poisson-Boltzmann equation and subsequent evaluation of the first moment of the counterion distribution before and after proton ejection by bR were performed. A contraction of the ion atmosphere caused by conversion of mono- to divalent TEMED cations is seen. This would give a negative component to the observed displacement currents. Involvement of the counterion atmosphere in the observed signals is strongly indicated by the abolition of the purported pump reversal when small amounts of divalent or larger amounts of monovalent cations are added and this is predicted in the calculation. Raising the TEMED concentrations gives the same effect, and in the same concentration range as observed in [9]. Thus, it is not necessary to conclude that TEMED reverses the direction of the proton pump.

2. RESULTS AND DISCUSSION

The PM is negatively charged due to both the amino acid side chains of bR and to the large fraction of the PM lipids which carry negative charges [10]. Transient release and subsequent binding of accumulated counterions with quantum yields significantly > 1 have been observed during the bR photocycle by measuring conductivity changes [11,12]. However, the large-scale nonproton ion release occurs at higher pH and higher ionic strengths [13] than used in [9], so only the effects of released protons have been considered below.

The counterion distribution near the PM can be calculated by solving the Poisson-Boltzmann equation which relates the potential at a point in space to the net charge distribution. This treats the counterions as a continuum of charge but since the PM fragments are so much larger and heavily charged than a simple ion, this approximation should be very good. The simplest case is to treat the PM as an infinite plane with a uniform charge per unit area. This problem has been analysed by Weisbuch and Gueron [14] for solutions containing both mono- and divalent cations. Their equations have been solved numerically. From the electrostatic potential obtained, the distribution of all charged species near the PM surface is easily calculated.

The critical point is that at the pH (6.9) used by Toth-Boconadi et al. [9], all of the diamines they cite are buffers since all have a pK near neutrality. Calculation of the expected distribution of released protons shows that for buffers with pK between 6 and 8, 93% or more of the protons will bind to the buffer species at pH 6.9 (see appendix in [12]). In particular, TEMED in 20 mM NaCl has a pK of 5.9 (not shown) and therefore at pH 6.9, TEMED will be primarily a $+1$ ion which protons released by bR will convert to the $+2$ ion. At low ionic strength, and at sufficiently low TEMED concentration, the quantity of protons released by the PM titrate enough of the TEMED buffer to produce an observable collapse of the counterion cloud toward the surface of the membrane. This would appear as a negative current relative to the direction of proton extrusion. The monoamines will not give a similar effect because their pK values are much higher and hence have drastically less buffer capacity than TEMED. In addition, protonation

of the miniscule concentrations of neutral monoamine at pH 6.9 will only produce a $+1$ ion, and this would be negligible compared to the concentration of monovalent cations present as the counterions for the charges on the PM.

These calculations assume that the divalent TEMED species can be treated as a simple divalent point charge since the Debye length in these dilute solutions is much longer than the distance between the two nitrogen atoms of TEMED. Alvarez et al. [15] present direct evidence that the quaternary ammonium equivalent of the TEMED $+2$ cation was well described as a simple divalent ion in experiments with phosphatidylserine vesicles and planar bilayers.

If the ion atmosphere shifts only a small distance, the net effect will be multiplied by the large quantity of charge moving and the observable currents are proportional to the product of the charge times the displacement. To quantitate this, the first moment of the excess charge distribution, $Q(X)$, near the PM surface was calculated. This is defined as $\langle x \rangle = \int Q(x) \cdot x dx / \int Q(x) dx$ where x is the distance from the PM. The dependence of the change in first moment before and after H^+ release has been calculated at different TEMED concentrations, or in the presence of mono- and divalent salts, to compare with the experimental results of [9].

As an independent check, integrals of the charge distribution were calculated, which by electrical neutrality should equal the surface charge density. In all cases, the integrals gave $> 97\%$ of the expected value. The calculations were done using surface charge densities of 0.4 and 1 e/nm^2 , which can be estimated from the PM structure [2] and lipid composition [10] and are similar to those given by Renthall and Cha [16].

Fig.1 shows the calculated charge distribution from 0 to 0.1-times the Debye length from the PM surface for bR suspended in water with 0, 2 and 20 μM added divalent ions. Most of the charge comes from the counterions, presumed monovalent, from the bR itself. Due to the high surface charge, the surface potential is several times kT and most of the counterions are located quite close to the surface. The distribution has a long tail which is not shown in fig.1: the counterion concentrations do not achieve their bulk value until approximately twice the Debye length. Note that low concentra-

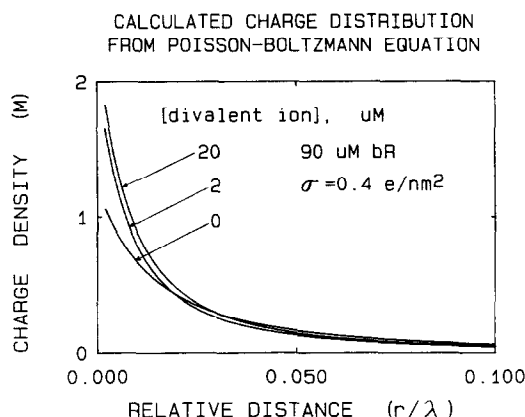


Fig.1. Calculated charge distribution as moles of electronic charges per liter vs the distance from the membrane in units of the Debye length. The three curves are calculated for 90 μM bR, using a surface charge of $0.4 \text{ e}\cdot\text{nm}^2$, for 0, 2 and 20 μM added divalent-monovalent salt, e.g. CaCl_2 . Note that the charge density in the immediate vicinity of the membrane is about twice as great in the presence of 20 μM divalent cation.

tions of added divalent ions give much more charge concentrated very close to the surface. Also, the long tail of the distribution is reduced. Addition of divalent ions thus produces an observable shift of the first moment of the charge distribution toward the membrane surface.

Table 1 gives the results for 90 μM bR suspended in 44 μM TEMED at pH 6.9 with various amounts of mono- or divalent cation salts added. The effect of the light flash is assumed to convert 20 μM TEMED +1 cation to the +2 form, corresponding to a quantum yield of 0.22 for H^+ release. The charge displacement is given for two values of the surface charge on the bR. Note that in the absence of added salt, the displacement of the charge cloud gives the equivalent of one electronic charge per bR moving about 1 nm in the direction opposite to the proton pump. If this is normalized to the amount of bR actively cycling (as is done in electrical measurements), it would yield a displacement of 3.5 nm. This is comparable to the displacements reported by Keszthelyi and Ormos [7] in the later stages of the photocycle. An order of magnitude estimate of the charge displacement produced by proton release in the absence of TEMED can be made with reference to fig.1: most of the charge is contained within 0.1-times

Table 1

Effect of added salt on counterion charge distribution before and after actinic flash

Concentration of added salt	Change in charge displacement from 1st moment, $\text{e}\cdot\text{nm}$ per bR ^a	
	$\sigma = 0.4 \text{ e/nm}^2$	$\sigma = 1 \text{ e/nm}^2$
Monovalent (mM)		
0	0.80	0.87
5	0.45	0.78
10	0.23	0.55
20	0.07	0.28
50	0.01	0.07
100	0.003	0.02
Divalent (μM)		
0	0.80	0.87
10	0.38	0.41
20	0.25	0.27
50	0.13	0.13
100	0.08	0.07
200	0.05	0.04
400	0.04	0.025

^a The moment calculated as $\langle x \rangle = \int Q(x) \cdot x dx / \int Q(x) dx$ where $Q(x)$ is the excess charge as a function of distance from the membrane surface, calculated as described in the text. This is then multiplied by the number of charges per bR, calculated from the known unit cell size of the PM [17] and the assumed charge density. The displacements are all negative, i.e. in the direction opposite to proton extrusion, and represent a partial collapse of the ion atmosphere toward the PM surface

Conditions: 90 μM bR, 44 μM TEMED, pH 6.9. Protons released after flash assumed to be 20 μM . The change listed is the difference in the moment of the charge distribution before and after the simulated flash, calculated for two values of the surface charge on the PM

the Debye length, 20 nm under these conditions. Assuming the quantum yield used above, the release H^+ would give a positive displacement of about $0.5 \text{ e}\cdot\text{nm}$ per bR in the sample. Addition of monovalent salt above 20 mM causes the counterion collapse to be effectively washed out. The same behavior is obtained by adding divalent cations, but the effect occurs at about 100-times lower concentrations. This is precisely the behavior seen in the data of fig.4 of [9].

Table 2

Concentration dependence of net charge displacement before and after actinic flash (90 μ M bR, pH 6.9)

[TEMED] (μ M)	Change in charge displacement from 1st moment, e \cdot nm per bR ^a	
	$\sigma = 0.4$ e/nm ²	$\sigma = 1$ e/nm ²
22	1.09	1.20
44	0.80	0.87
60	0.68	0.74
80	0.58	0.63
100	0.51	0.54
150	0.40	0.41
200	0.33	0.33
300	0.25	0.25
500	0.18	0.17

^a Displacements calculated as in table 1. All displacements are negative, i.e. opposite to the direction of proton extrusion

Since TEMED itself is a mixture of mono- and divalent cations, the results above suggest that simply raising the TEMED concentration should give the same effect. Table 2 gives the calculated displacements as a function of [TEMED] at pH 6.9. At only 100 μ M TEMED, the change in the displacement following proton extrusion is reduced by about half. As above, this is exactly the concentration range where Toth-Boconadi et al. observed abolition of the supposed reversal of the proton pump. Also, in both tables 1 and 2, there is little dependence on the surface charge, which is the only adjustable parameter in the calculation.

From the data shown in [9], the time scale where differences are observed in the electrical signals with or without TEMED is about 20 μ s. Neither diffusion of the ion cloud nor equilibration of the buffer ions can be rate-limiting: a small ion will diffuse 10 nm in 50 ns and the protonation of the buffer should be complete in < 1 μ s in the region near the PM where the ions are highly concentrated. Therefore, the counterion cloud will track H⁺ release by the PM and on the longer time scales counterion collapse will be indistinguishable from other charge movements associated with the bR photocycle.

The effect of the diamines observed by Toth-Boconadi et al. [9] can therefore be explained by displacements of the counterion atmosphere near the PM. The experimental conditions used were

fortuitously just the right combination of concentration and buffer species where it was possible to observe a change in the counterion distribution following proton extrusion by bR. In itself it is a very intriguing – perhaps unique – observation, but does not require one to conclude that the direction of the pump has been reversed by TEMED. The calculations presented here also illustrate the importance of counterion effects with bR, particularly at the low ionic strengths often employed experimentally.

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